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Supporting Information

Semisynthetic Maturation of [FeFe]-Hydrogenase using $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$: Key Roles for HydF and GTP

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Experimental Methods

General Procedures

NMR spectra were recorded at 298 K on a Bruker ASCEND AVANCE III HD 500 MHz (500.2 MHz for ^{1}H) spectrometer equipped with a Prodigy (liquid nitrogen cooled) cryoprobe or a Bruker AVANCE NEO 400 MHz (400.1 MHz for ^{1}H NMR spectroscopy) spectrometer. ^{1}H NMR chemical shifts (δ in ppm) are referenced to residual ^{1}H solvent resonances in the deuterated solvent. All reagents were purchased from commercial sources and used as received. The solvents used for synthetic procedures (tetrahydrofuran (THF), diethyl ether, acetonitrile, methanol, and n-pentane) were sparged and stored under Ultra High Purity (UHP) argon gas before being dried via passage through a CHEMBLY solvent purification system (formerly JC Meyer Solvent Systems) using UHP argon as the working gas. All glassware was heated to 160 °C overnight prior to use.

Synthesis and Materials

All synthetic procedures were performed under an atmosphere of N_2 or Ar using standard glovebox or Schlenk line techniques unless otherwise noted. $Fe_3(CO)_{12}$ was purchased from Acros Chemical. Potassium cyanide was purchased from Sigma Aldrich. 18-crown-6 was purchased from Oakwood Chemical. H_2S gas was supplied by Praxair and used as received. CD_3CN was purchased from Cambridge Isotope Labs, degassed by three freeze-pump-thaw cycles, and stored in an argon filled glovebox over activated molecular sieves. $K[Fe(CN)(CO)_4]$ was prepared from $Fe_3(CO)_{12}$ according to the published literature procedure.[1]

Preparation of $[K_2(18\text{-crown-}6)_2(thf)][Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]$

$[K_2(18\text{-crown-}6)_2(thf)][Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]$, a source of $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$ or $[2Fe]_E$, was prepared from $K[Fe(CN)(CO)_4]$ according to the published literature procedure[1] with the following adaptations: Two 370 nm (Gen 1) Kessil LED lamps were used in place of the 365 nm light source noted in the published procedure to irradiate the reaction mixture while H_2S was purged through the reaction mixture. Recrystallization of $[K_2(18\text{-crown-}6)_2(thf)][Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]$ was achieved by dissolving the crude dark-orange product in a minimal amount of acetonitrile (ca. ~1 mL), followed by layering with 3 mL of THF and 4 mL Et_2O. A dark-red crystalline solid was isolated after storing the layered solution at -30 °C overnight in the glovebox freezer. Crystalline yield: 70 mg (6%). ^{1}H NMR spectral data recorded in CD_3CN matched the reported literature values.[1]

Expression and purification of proteins

The expression, isolation and reconstitution of strep-tagged and his-tagged *Clostridium acetobutylicum* (*C.a.*) HydF, and His-tagged HydA from *Chlamydomonas reinhardtii* (*C.r.*) followed previously published protocols:, *Ca*HydF[2], and *Cr*HydA.[3, 4]

Expression of T-protein, SHMT, and H-protein were as previously described.[4, 5] Synthesis of H_{met} from H_{red} was as previously described.[4]

Size Exclusion Chromatography

HydF was analyzed via gel filtration on an FPLC (ÄKTA Pure) using a HiPrep 16/60 Sephacryl S-200 High Resolution column equilibrated with anaerobic 50 mM HEPES pH 7.5, 250 mM KCl buffer that was supplemented with 0.5 mM DTT and 0.5 mM DT inside a Coy chamber. The HydF

sample (32 μM monomeric concentration, 3.2 ± 0.3 Fe/monomer) was incubated with 1 mM DTT and 1 mM DT for 30 min prior to being injected onto the S-200 column. A separate sample of HydF was incubated with a 7.3-fold excess of $[2Fe]_E$ in the presence of 1 mM DTT and 1 mM DT for 30 min in the dark prior to being injected onto the S-200 column. Both sample runs were followed by monitoring the absorbance at 280 nm.

Photolysis of HydF/$[2Fe]_E$

Select samples of $[2Fe]_E$/HydF were photolyzed in order to confirm the presence of CO in the enzyme-compound complex using H64L deoxymyoglobin (deoxyMb) as a reporter.[3] Samples for photolysis were prepared in an anaerobic MBraun chamber ($O_2 \leq 0.5$ ppm) and contained $[2Fe]_E$/HydF in 50 mM HEPES, 250 mM KCl, 5% glycerol, pH 7.5 buffer that was supplemented with 2.5 mM DT. HEPES buffer, H64L Mb and DT were combined and transferred to a 1 mm or 10 mm path-length screw top (anaerobic) cuvette (Starna Cells Inc., Atascadero, CA, USA). UV-vis absorbance spectra were recorded from 200 -800 nm using a benchtop Cary 60 spectrophotometer with a 0.5 nm data interval. The cuvette was then transferred back to the MBraun chamber and $[2Fe]_E$/HydF was then added to the mixture. Samples were then removed from the MBraun and exposed to a 300 Watt xenon lamp to initiate photolysis. The spectral characteristics were monitored periodically until the changes to the Soret band from deoxyMb (432 nm) to carboxyMb (425 nm) stabilized (1-2 hours).

EPR Sample Preparation

X-band EPR samples were prepared inside an MBraun glovebox operated with a 100% N_2 atmosphere (O_2 levels ≤ 1 ppm). Samples of HydF were prepared in the presence of 2 mM DTT and 2 mM DT and were transferred into EPR tubes (Wilmad LabGlass, 4 mm OD, NJ, USA). Tubes were then capped with septa, incubated for 10 – 15 minutes in the dark, and then were removed from the chamber and immediately flash frozen in liquid N_2. Samples were stored in liquid N_2 until spectral acquisition occurred.

***In vitro* Activation Assays**

The *in vitro* activation assays were performed as described previously with modifications.[3, 4] *In vitro* maturations of *Cr*HydA were carried out at ambient temperature in an anaerobic MBraun chamber ($O_2 \leq 1$ ppm). A standard reaction consisted of 40 μM HydF, 4 μM HydA, 40 μM or 500 μM $[K_2(18\text{-crown-}6)_2(thf)][Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]$, 2 mM L-cysteine, 1 mM DTT, 2 mM DT, 20 mM GTP, and 6.4 mM Fe^{2+} as ferrous sulfate, 1 mM PLP, 10 μM T-protein, 5 μM SHMT, 44 mM L-serine, and 47 mM NH_4Cl in 50 mM HEPES pH 7.5, 150 mM KCl buffer. As previously reported, in reactions containing the T-protein, addition of the H-protein was no longer necessary due to co-purification of the H-protein with the T-protein.[4, 6]

To prepare the hydrogenase activity assay, 2 μL of the reaction mixture was diluted to 2 mL (final volume) using 50 mM Tris pH 6.9 buffer; DT and methyl viologen were then added to the mixture to final concentrations of 20 mM and 10 mM, respectively. After 3 to 30 minutes, headspace gas (100 μL) was removed from the vial with a Hamilton gas tight syringe. H_2 production was quantified using a SHIMADZU GC-2014 with a TCD detector using N_2 as a carrier gas.

EPR Spectroscopy

Low temperature continuous wave (CW), X-band (9.38 GHz) EPR spectra were collected using a Bruker EMX spectrometer fitted with a ColdEdge (Sumitomo Cryogenics) 10 K waveguide in-cavity cryogen free system, Oxford Mercury iTC controller unit, and helium Stinger recirculating unit (Sumitomo Cryogenics, ColdEdge Technologies, Allentown, PA). Helium gas flow was maintained at 100 psi. Unless otherwise noted, spectral parameters for data collection were: 12.0 K, 1.0 mW microwave power, 100 kHz modulation frequency, 10 G modulation amplitude, and spectra were averaged over 4 scans. Spectral data were plotted using the software program OriginPro (2018b, OriginLab Corp. Northampton, MA, USA), and all spectral data were baseline and cavity corrected.

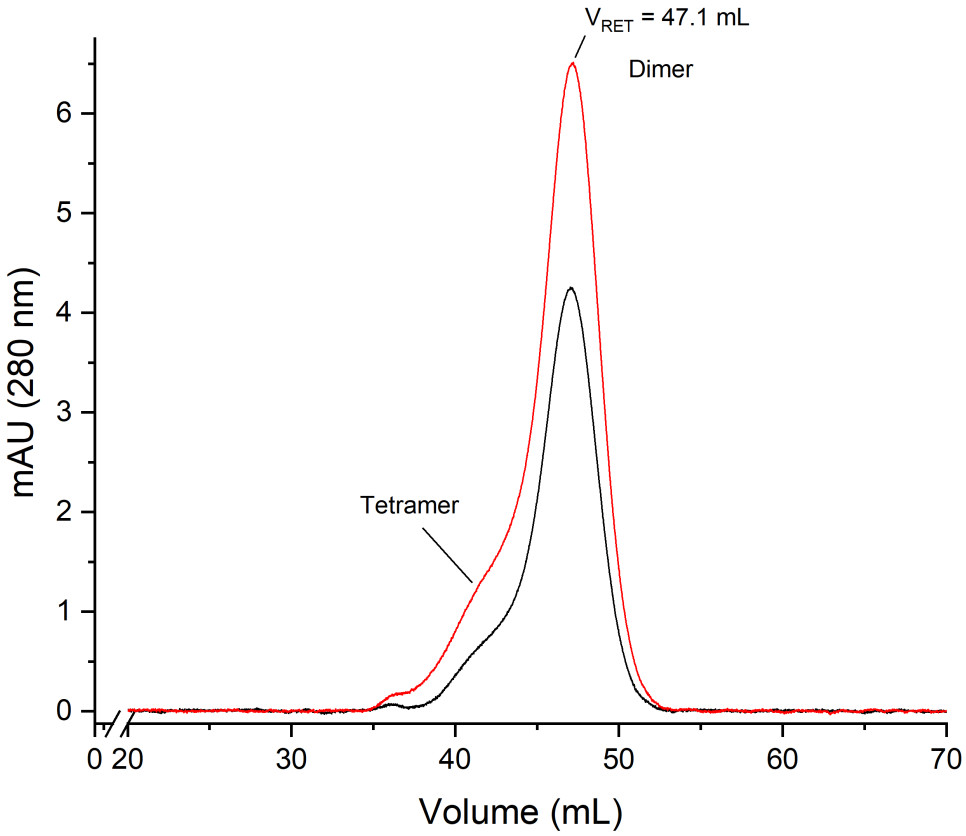

Figure S1. Gel filtration of *C.a.* HydF incubated with synthetic $[2Fe]_E$; HydF alone (black) or HydF preincubated with excess $[2Fe]_E$ (red).

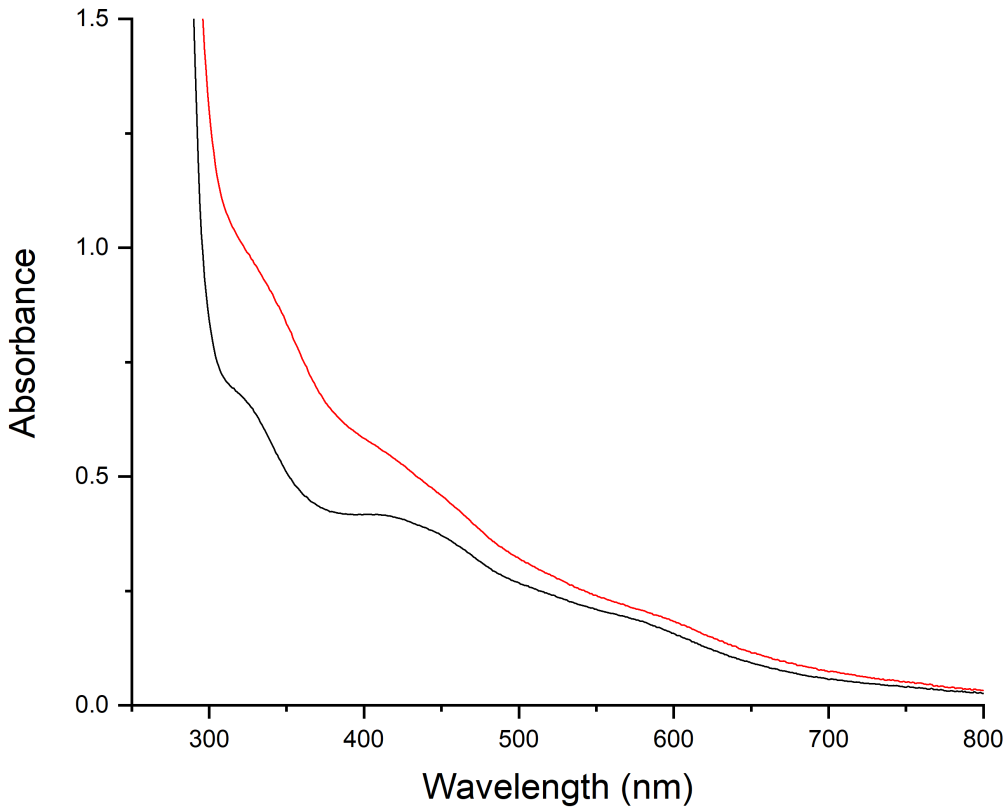

Figure S2. An aliquot of HydF (3.2 ± 0.3 Fe/monomer) was thawed in the MBraun chamber, diluted into 50 mM HEPES, pH 7.5, 250 mM KCl, 5% glycerol buffer and then analyzed via UV-Vis (black spectrum). This sample was then transferred back to the MBraun chamber and a 2-fold excess of $[2Fe]_E$ was added and allowed to incubate with the protein for 15 min in the dark and in the absence of reducing agents. The sample was then subjected to several buffer exchange events using the aforementioned buffer and Amicon spin filters (MWCO 30 kDa). The final product was then analyzed via Bradford, AA, and UV-Vis (red); the protein was determined to be 45.7 μM monomer with 4.45 ± 0.05 Fe/monomer. The control spectrum (black) was normalized to this same protein concentration and then a difference spectrum was made (see **Figure 1**).

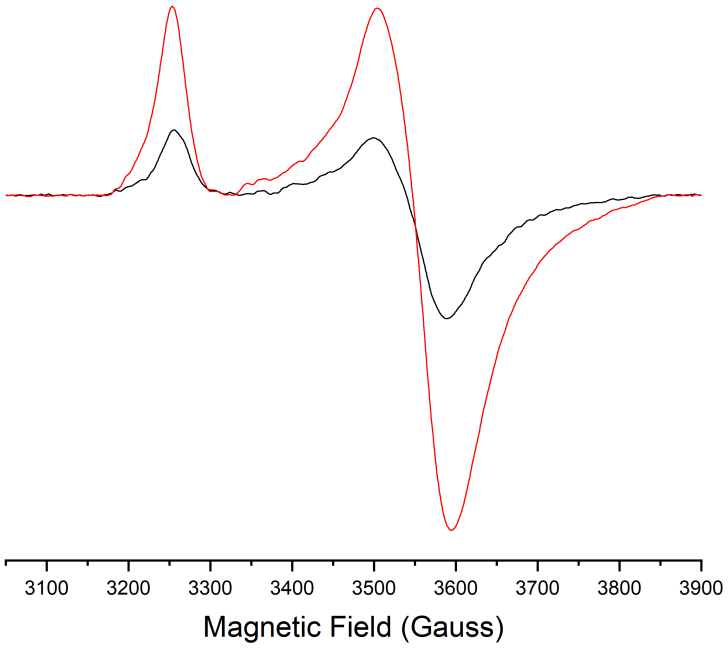

Figure S3. A control sample of HydF (24 μM monomeric concentration, 3.2 ± 0.3 Fe/monomer) was reduced with 2 mM DTT and 2 mM DT for 10 min before being flash frozen (black). A separate sample of HydF (3.2 ± 0.3 Fe/monomer) was reduced with 2 mM DTT and 2 mM DT and was then treated with a 3.5-fold excess of $[2Fe]_E$ prior to undergoing several buffer exchange events using Amicon spin filters (final product 24 μM monomeric protein with 5.1 ± 0.2 Fe/monomer).

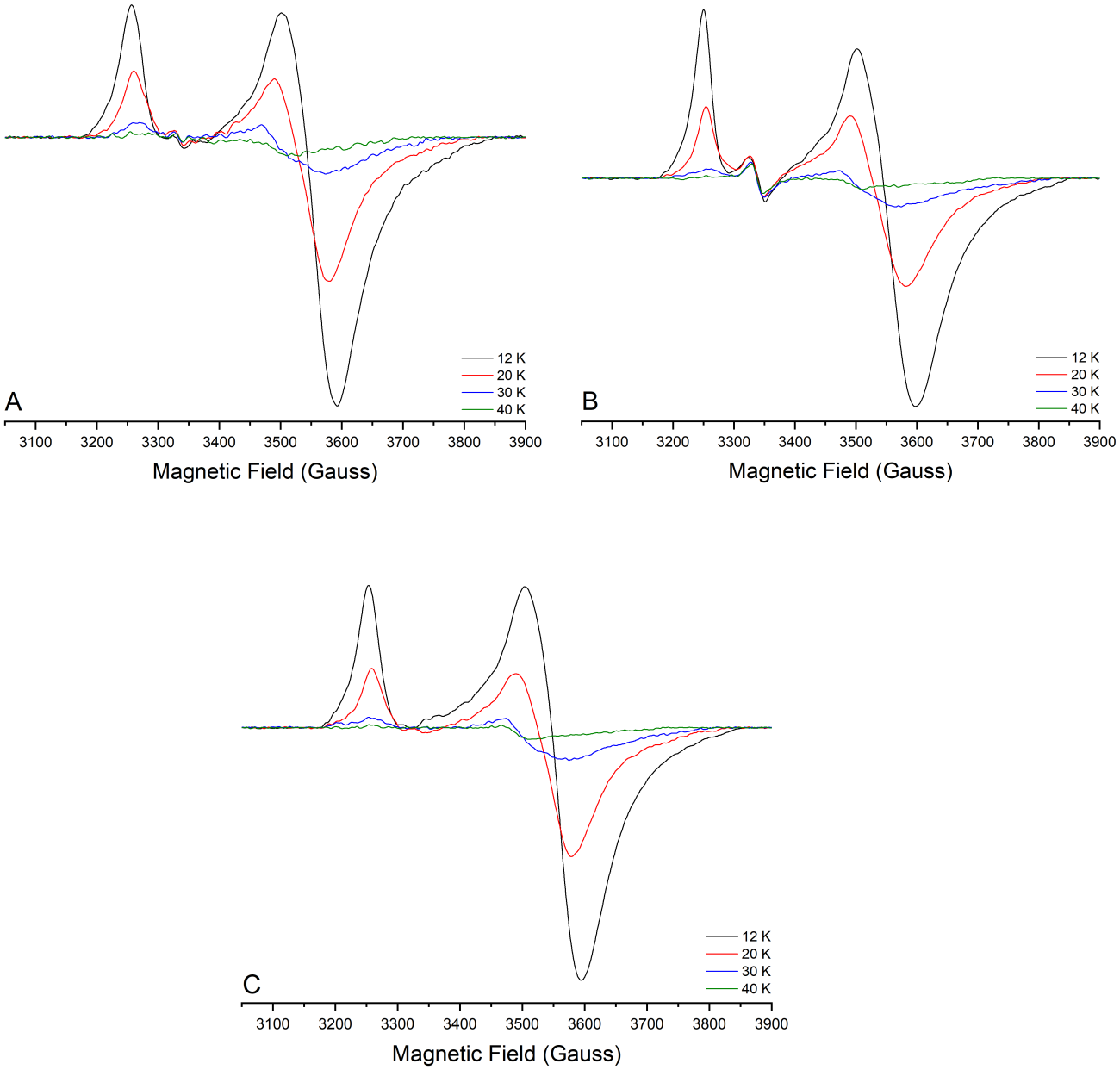

Figure S4. HydF temperature dependent EPR spectroscopy of HydF ± $[2Fe]_E$. **A**. Control HydF (40 μM monomeric concentration, 3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT (2 hr time point). **B**. HydF (40 μM monomeric concentration, 3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT in the presence of a 7.3-fold excess of $[2Fe]_E$ (2 hr time point). **C**. A sample of HydF (3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT and treated with a 3.5-fold excess of $[2Fe]_E$ prior to undergoing buffer exchange using Amicon spin filters (final product 24 μM monomeric protein with 5.1 ± 0.2 Fe/monomer).

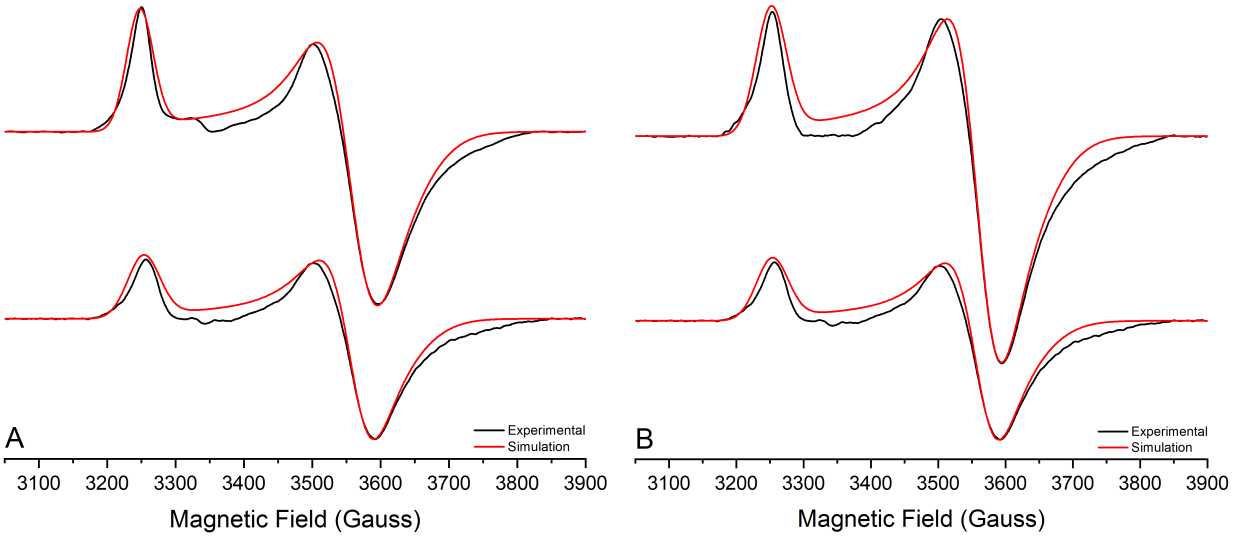

Figure S5. EPR simulation results. **A**. *Bottom*. Control sample of HydF (3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT (g = 2.058, 1.879, 1.862; g-strain = 0.035, 0.033, 0.072). *Top*. HydF (3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT in the presence of a 7.3-fold excess of $[2Fe]_E$ (g = 2.062, 1.879, 1.858; g-strain = 0.028, 0.037, 0.072). **B**. *Bottom*. Control sample of HydF (3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT (g = 2.058, 1.879, 1.862; g-strain = 0.035, 0.033, 0.072). *Top*. A sample of HydF (3.2 ± 0.3 Fe/monomer) reduced with 2 mM DTT and 2 mM DT and treated with a 3.5-fold excess of $[2Fe]_E$ prior to undergoing buffer exchange using Amicon spin filters (g = 2.059, 1.878, 1.857; g-strain = 0.033, 0.033, 0.071). The final product was determined to have an iron number of 5.1 ± 0.2 Fe/monomer.

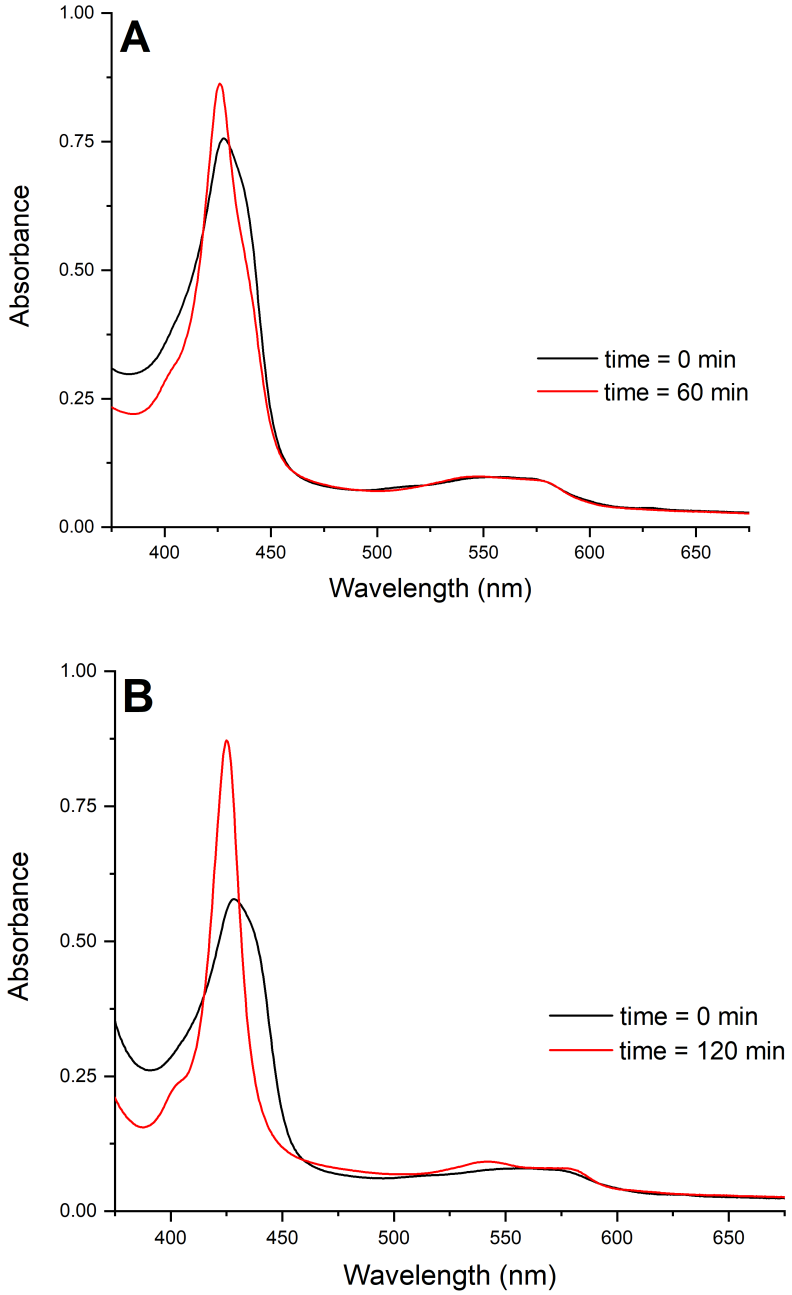

Figure S6. UV-visible spectroscopic characterization of Mb H64L before (black) and after (red) photolysis of $[2Fe]_E$/HydF with a xenon lamp in the presence of Mb H64L. **A**. $[2Fe]_E$/HydF (8.5 µM) and Mb H64L (47.5 µM heme) in a 1 mm cuvette illuminated for 60 min gives 15 µM CO detected. **B**. $[2Fe]_E$/HydF (2 µM) and Mb H64L (4 µM heme) in a 1 cm cuvette illuminated for 120 min gives 3.9 µM CO detected.

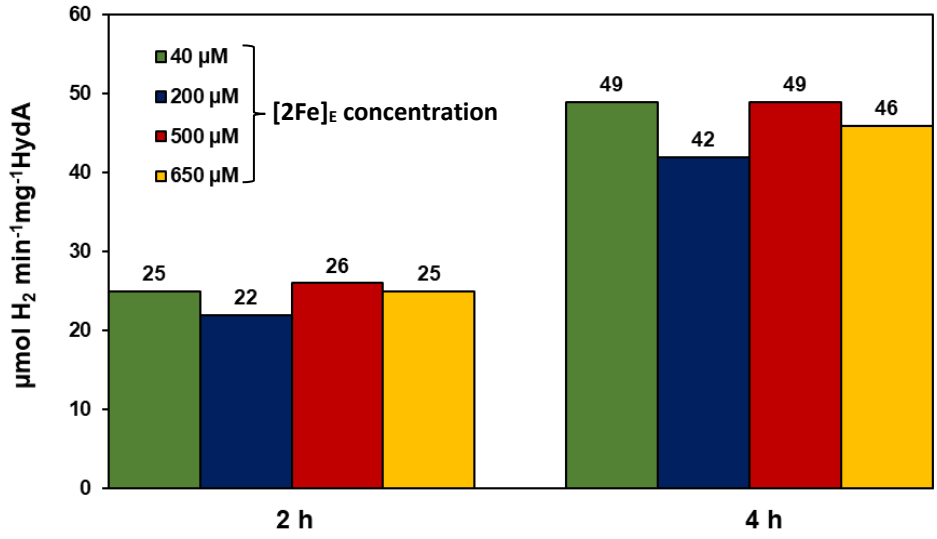

Figure S7. Dependence of HydA maturation on the concentration of $[2Fe]_E$. HydA was matured as described in the methods but with two different concentrations of $[2Fe]_E$. The presence of excess $[2Fe]_E$ over HydF (40 µM in these assays) does not improve HydA maturation, supporting the idea that a 1:1 $[2Fe]_E$/HydF complex is the relevant species during maturation of HydA.

SI References

1. Y. Zhang, L. Tao, T. J. Woods, R. D. Britt and T. B. Rauchfuss, *J. Am. Chem. Soc.*, 2022, **144**, 1534-1538.
2. E. M. Shepard, A. S. Byer, J. N. Betz, J. W. Peters and J. B. Broderick, *Biochemistry*, 2016, **55**, 3514-3527.
3. E. M. Shepard, S. Impano, B. R. Duffus, A. Pagnier, K. S. Duschene, J. N. Betz, A. S. Byer, A. Galambas, E. C. McDaniel, H. Watts, S. E. McGlynn, J. W. Peters, W. E. Broderick and J. B. Broderick, *Dalton Trans.*, 2021, **50**, 10405-10422.
4. A. Pagnier, B. Balci, E. M. Shepard, H. Yang, D. M. Warui, S. Impano, S. J. Booker, B. M. Hoffman, W. E. Broderick and J. B. Broderick, *Angew. Chem. Int. Ed.*, 2022, **61**, e202203413.
5. R. M. Cicchillo, D. F. Iwig, A. D. Jones, N. M. Nesbitt, C. Baleanu-Gogonea, M. G. Souder, L. Tu and S. J. Booker, *Biochemistry*, 2004, **43**, 6378-6386.
6. J. Bourguignon, M. Neuburger and R. Douce, *Biochem. J.*, 1988, **255**, 169-178.